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STUDY ON THE OCCURRENCE OF *BORRELIA BURGDORFERI SENSU LATO* AND TICK-BORNE ENCEPHALITIS VIRUS (TBEV) IN TICKS COLLECTED IN LUBLIN REGION (EASTERN POLAND)

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Abstract: In 114 *Ixodes ricinus* ticks from 1 district of Lublin region (eastern Poland) examined by dark field microscopy method, the presence of motile spirochetes, morphologically corresponding to *Borrelia* species was detected in 8.8% of the total examined ticks. The highest infection rate was noted among females (16.7%), much lower in males (7.1%) and nymphs (4.0%). Examination of 550 ticks collected from 3 districts of the Lublin region by polymerase chain reaction (PCR) showed that 5.3% of the total number of ticks were infected with *Borrelia* spirochetes. The highest rates of *Borrelia* infection were observed in *Ixodes ricinus* ticks from the Zamość and Lublin districts (9.6% and 4.7% respectively). In the Włodawa district, only 2.4% ticks showed the presence of *Borrelia* DNA. In contrast to the results obtained by dark field microscopy method, the highest infection rate was noted in males (11.2%), followed by females (6.9%) and nymphs (1.7%). 57 *Ixodes ricinus* ticks collected from 3 Lublin districts (Lubartów, Lublin, Radzyń Podlaski) were tested for the presence of tick-borne encephalitis virus (TBEV) by the inoculation of 5-week-old Swiss mice, followed by blind passages and inoculation of GMK cell culture with brain suspension of the infected mice. One strain of TBEV was isolated from a pool of 24 *I. ricinus* ticks collected from the Radzyń Podlaski district. The minimum infection rate of ticks from this district with TBEV was estimated as 4.2%, and in the total area of Lublin region estimated as 1.8%.

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INTRODUCTION

The determination of the proportion of ticks infected with *Borrelia burgdorferi* and tick-borne encephalitis (TBE) virus (TBEV) on a particular territory is very important for evaluation of the risk of acquiring a disease transmitted by ticks, in particular for people occupationally exposed to tick bites [3, 5, 6, 18, 19, 33]. The detection of *B. burgdorferi* bacteria or TBE virus together with observed clinical cases provides a basis for considering the area under study as an endemic region of borreliosis and/or tick-borne encephalitis [5, 6, 10, 18, 33].

The diseases transmitted by ticks, especially borreliosis, represent a growing health problem in Poland, which also concerns the Lublin region [3, 4, 5, 6]. However, the spread of the vector ticks *Ixodes ricinus* and their

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infection rate with both *Borrelia* spirochetes and TBE virus have not been sufficiently determined. Accordingly, the objective of this study was a preliminary determination of the infection of ticks collected in the Lublin region with the above-mentioned pathogens.

MATERIALS AND METHODS

Areas of study and sampling methods. Unfed ticks (adults and nymphs) belonging to the species *Ixodes ricinus* were collected during spring/summer season in the years 2000–2001 in 5 districts of the Lublin region (eastern Poland) (Fig. 1) by flagging lower vegetation at peripheral areas and inner parts of deciduous and mixed forests, including suburban localities. Ticks were collected at the edge of forests and farmers' fields, along the forest paths and in the surroundings of parking lots or picnic areas. Collected ticks were examined with 3 different methods as described below, 2 major lots for the presence of *Borrelia burgdorferi* and 1 lot for the presence of TBE virus:

- 114 ticks from the Lublin district collected in May-June 2000 were examined by dark field microscopy for the presence of *B. burgdorferi*.
- 550 ticks collected from 3 districts of the Lublin region (Lublin, Zamość, Włodawa) during the period April-June 2001 were placed in vials separately (adults), or in pools of 5 specimens (nymphs), killed with hot water and immersed in 70% ethanol for further investigation by polymerase chain reaction (PCR) for the presence of DNA of *B. burgdorferi*.
- 57 adult ticks (31 males and 26 females) collected in May-June 2000 from the districts of Radzyń Podlaski (24 specimens), Lubartów (14 specimens), and Lublin (19 specimens) were checked for the presence of TBE virus by inoculation of 5-week-old mice (Swiss) with tick suspension, followed by "blind" passages of mice brain suspension and cultivation in GMK cell culture.

Examination of ticks for the presence of *Borrelia budgdorferi sensu lato* by dark field microscopy. 114 ticks collected in 2000 from the Lublin suburban localities (Dąbrowa, Prawiedniki, Bychawka), after removal from the cloth and rinsing in 40% ethanol and next in PBS, were examined for motile *Borrelia* spirochetes according to Wilske [34], by the observation of extracts from tick's mitgut in BSKH liquid medium (Sigma), in a dark microscopic field (× 312, Jenamed 2, Germany). Adult ticks were examined singly and nymphs in pools of 5 specimens. Minimum infection rate was calculated according to Kahl [12].

Examination of ticks for the presence of DNA of *Borrelia burgdorferi sensu lato* by polymerase chain reaction (PCR). 550 ticks collected from the districts of Lublin (362 specimens), Włodawa (84 specimens) and Zamość (104 specimens) were analysed for *Borrelia* infection by polymerase chain reaction (PCR). DNA was isolated from the examined ticks by boiling in 0.7 M ammonium hydroxide [23]. After removal from ethanol, all specimens were dried on tissue paper and then each adult individually and nymphs in pools of 5 or 6 specimens were immersed in 0.7 M NH_4OH and crushed with pipette tips. The suspensions were boiled at 98ºC for 15–20 min in a heating block in 2 ml Eppendorf vials. Then vial caps were opened and heating was extended for another 15 min to remove ammonia and reduce volume to 50 µl. The lysates (tick DNA) were stored at -20ºC.

Figure 1. The area of study. Shadowed area: The territory of Lublin region. Circles: main towns of districts.

PCR was performed with the use of:

- Complementary primers for known *fla* gene sequences of *Borrelia bugdorferi sensu stricto*, *Borrelia garinii*, and *Borrelia afzelii* (produced by DNA, Gdańsk, Poland).
- Hyperthermostable polymerase (Delta 2, DNA, Gdańsk).
- DNA reference standard from 53–1031 base pairs (MBI Fermentas, Lithuania).
- Mixture of dnTP nucleotides (DNA, Gdańsk).
- Buffer loading for samples during electrophoresis (MBI Fermentas, Lithuania).
- Genomic DNA *Borrelia burgdorferi sensu stricto* as a positive control (DNA, Gdańsk).
- Negative control (re-distilled water).

The size of the amplified fragment was 442 base pairs. Amplification products were identified in 1.5% agarose gel, after electrophoresis performed in standard conditions and staining with ethidium bromide (Fig. 2). The results of the reaction were visualised by UV transillumination in ultraviolet light.

Examination of ticks for the presence of TBE virus. 57 ticks collected in the following districts: Radzyń Podlaski (24 ticks, one pool), Lubartów (14, one pool), Lublin (19, two pools) were checked for the presence of

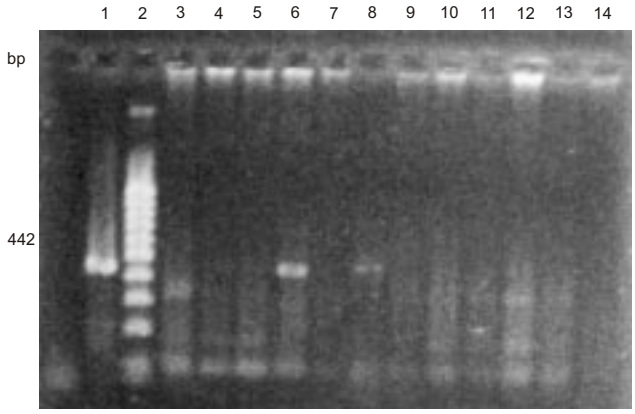

Figure 2. PCR amplification of *Borrelia burgdorferi sensu lato* in *I. ricinus* extracts demonstrated by agarose gel electrophoresis after ethidium bromide staining. Lane 1: Positive control (*B. burgdorferi* genomic DNA); Lane 2: Marker (53-1031 base pairs); Lane 3-5, 7, 9-13: Tick midguts with negative amplification; Lane 6, 8: Tick midguts with positive amplification; Lane 14: Negative control.

TBE virus (TBEV). The ticks collected in particular localities were merged in pools of 8–24 specimens, homogenized and suspended in PBS with an admixture of 10% horse serum and antibiotics (100 units/ml penicillin and 2 mg/ml streptomycin). After centrifugation, supernatant was administered to 5-week-old mice (Swiss) intracerebrally (0.02–0.03 ml). The extract from a single tick pool was administered on average to 4 mice. The control group consisted of 4 mice not receiving the extract. The reactions and behaviour of the animals were observed daily for 14 days and thereafter the mice were anaesthetized. Then, 3 blind passages were performed every 7 days with brain suspensions of the infected and control mice. The fourth passage was conducted only if the occurrence of neurological symptoms in the mice was observed. Then, brain suspensions of inoculated mice were transferred to GMK culture. As the viruses of tick-borne encephalitis do not cause a clear cytopathic effect in cell cultures [2, 9, 20], TBE virus was detected by an indirect method based on the phenomenon of virus interference. As an auxiliary model ECHO 9 virus was used, which induces cytopathic effect in GMK culture. If the cytopathic effect of ECHO 9 virus was delayed in the presence of examined suspension, it was assumed that this suspension contained TBE virus. The identity of the isolated TBE virus was confirmed serologically. The viral antigen was prepared from the infected GMK culture according to literature data [7, 20] in own modification. Antigen was fixed to standard ELISA microplates and tested in the ELISA assay with TBE-positive and TBE-negative human sera produced by PROGEN Biotechnik GmbH (Heidelberg, Germany). The test was performed by the modified procedure of PROGEN Biotechnik GmbH and the extinction was read on a microelisa reader at wavelength 450 nm. The minimum infection rate of ticks with TBEV was calculated on the basis of an assumption that in the case of positive virus isolation only 1 tick in the examined pool was infected.

Table 1. Infection rates of adult and nymphal *Ixodes ricinus* ticks with *Borrelia burgdorferi sensu lato* in Lublin district, determined by dark field microscopy.

Stage	No. examined	No. positive	%
Females	36	6	16.7
Males	28	2	7.1
Nymphs*	50	2	4.0**
Total	114	10	8.8

*examined in pools; **minimum infection rate.

Table 2. Infection rates of adult and nymphal *Ixodes ricinus* ticks with *Borrelia burgdorferi sensu lato* in selected forested areas of 3 districts in Lublin region determined by the PCR results.

District	Stage	No. examined	No. positive	%
Lublin	Females	103	9	8.7
	Males	79	5	6.3
	Nymphs*	180	3	1.7**
	Subtotal	362	17	4.7
Włodawa	Females	10	0	0
	Males	48	2	4.2
	Nymphs*	26	0	0
	Subtotal	84	2	2.4
Zamość	Females	18	0	0
	Males	26	10	38.5
	Nymphs*	60	0	0
	Subtotal	104	10	9.6
Total	Females	131	9	6.9
	Males	153	17	11.2
	Nymphs*	266	3	1.1**
	Total	550	29	5.3

*examined in pools; **minimum infection rate.

RESULTS

Prevalence of *Borrelia burgdorferi sensu lato* in ticks determined by dark field microscopy. The presence of motile spirochetes, morphologically corresponding to *Borrelia* species was detected in 8.8% of the total examined ticks. The highest infection rate was noted among females (16.7%), then in males (7.1%) and in nymphs (4.0%) (Tab.1).

Prevalence of *Borrelia burgdorferi sensu lato* in ticks determined by the polymerase chain reaction (PCR). Examination of 550 ticks by polymerase chain reaction (PCR) showed that 5.3% of the total number of ticks in the study were infected with *Borrelia* spirochetes. The highest rates of *Borrelia* infection were observed in ticks collected from the Zamość and Lublin districts (9.6% and 4.7% respectively). In the Włodawa district, only 2.4% ticks showed the presence of *Borrelia* DNA. In contrast to the results obtained by dark field microscopy, the highest

Table 3. Recovery of TBE virus in mice infected with suspensions of ticks.

District	No. of pools	No. of ticks in the pool	No. of infected mice	Occurrence of symptoms in consecutive passages (number of symptomatic mice)				Minimum infection rate (%)
				1st passage	2nd passage	3rd passage	4th passage	
Radzyń	1	24	4	0	0	1	1*	4.2%
Lubartów	1	14	4	0	0	0	ND	0
Lublin (A)	1	8	4	0	0	1	0	0
Lublin (B)	1	11	4	0	0	0	ND	0
Total	4	57						1.8%

*positive isolation confirmed in the GMK tissue culture after inoculating with ECHO 9 virus; ND = not done.

infection rate was noted in males (11.2%), followed by females (6.9%) and nymphs (1.7%) (Tab.2).

Prevalence of tick-borne encephalitis virus in ticks from 3 districts of Lublin region. Pathological symptoms (contracture of the lower extremities) resembling paralytic symptoms were noted in third blind passage in the mice lines infected with the suspensions of ticks from the areas of Radzyń Podlaski and Lublin (A) (Tab. 3). In fourth passage, in only 1 of 4 mice of the line infected with the suspension of ticks collected in the area of Radzyń Podlaski occurred symptoms similar to those in third passage (Tab. 3). In the GMK culture infected with the brain suspension of this mouse, the cytopathic effect of ECHO 9 virus was delayed, compared to the control virus. Based on this result it was assumed that tick-borne encephalitis virus was present in the examined material. The identity of the isolated TBE virus was confirmed by the serological examination.

The minimum infection rate of ticks from the Radzyń Podlaski district with TBEV was estimated as 4.2% and in the total area of Lublin region estimated as 1.8%.

DISCUSSION

Peťko *et al.* [17] calculated the minimum infection rate with *Borrelia burgdorferi* in ticks from the territory of Southern Poland by dark field microscopy method. The infection rate for females was 10.3%, for males - 9.8%, and for nymphs - 8.2%. Siuda *et al.* [26] observed 8.1% of infected nymphs and 10.8% of infected adults among ticks collected from the urban areas of Lublin.

Studies on distribution of *Borrelia burgdorferi sensu lato* in *Ixodes ricinus* ticks by the use of PCR method were conducted in various regions of Poland [10, 16, 27, 28]. The highest infection rate with *Borrelia* spirochetes was observed in ticks collected from the Katowice (Upper Silesia) region (37.5%), then in the Poznań (Wielkopolska) region (24.5%). In other Polish regions, such as Bydgoszcz (Kujawy), Kraków (Małopolska), Szczecin (Western Pomerania) and Olsztyn (Masurian Lakes region), the percent of infected ticks fluctuated from 7.4% in Bydgoszcz region to 15.5% in the Kraków region [10, 24, 27, 28]. Siński and Pawełczyk [24] concluded that in the Masurian Lakes territory adult forms of ticks are much more infected with *Borrelia* bacteria (31.0%) compared to nymphs (7.1%). Jenek and Głazaczow [10] in the area of Poznań (Wielkopolska) region observed 28.8% of infected females, 22.0% of infected males and 8.3% of infected nymphs. Nowosad *et al.* [16] found in ticks collected in the same area 1 year later the highest infection rate with *Borrelia* in nymphs collected during spring (28.6%), followed by females (21.2%) and males (10.7%). Similar infection rates in males and females were reported by Skotarczak and Wodecka [27] in ticks collected in the area of the Szczecin region - 12.3% and 12.1% respectively. Stańczak *et al.* [28] examined for the presence of *B. burgdorferi* 310 *I. ricinus* ticks collected in the Lubartów district of the Lublin region and found the highest infection rate in males (33.3%) followed by females (27.3%) and nymphs (1.9%).

In other European countries, Štépánová-Tresová *et al.* [29] examined 163 *I. ricinus* ticks from the České Budějovice region (Czech Republic) for the presence of *Borrelia* spirochetes by dark field microscopy method in 2 consecutive years and obtained 11.0% and 4.8% of positive results. In another paper, Štépánová-Tresová *et al.* [30] found by the same method that in the Košice area (Slovak Republic) the percentages of positive ticks in 4 consecutive years 1994–1997 amounted to 4.8%, 17.2%, 15.5% and 14.2%, respectively.

Recently, Barral *et al.* [1] found by the PCR test in Spain (Basque Country) 9.3% of infected adult ticks and 1.5% of infected nymphs among the total number of 1,535 examined *I. ricinus* specimens. Rauter *et al.* [22] noted 40% of positive adult ticks out of 548 examined and 30% of positive nymphs out of 507 examined from the Konstanz area (Germany).

The total infection rates of *Ixodes ricinus* ticks found in the present work by the dark field microscopy and PCR methods were 8.8% and 5.3% respectively, being within the range reported by other authors [1, 15, 26, 28, 29, 30]. The results of these 2 methods are not fully comparable, as they were obtained in different years. Nefedova *et al.* [15] reported a lack of correlation between the PCR and

dark field microscopy methods when examining *Ixodes persulcatus* ticks for the presence of *Borrelia burgdorferi*.

It is noteworthy that in the present work the percentage of infected *I. ricinus* adults was much higher than the percentage of infected nymphs. It corresponds to the results obtained by the majority of other authors [1, 10, 17, 22, 24, 28, 30].

In conclusion, the results of this study show that a considerable proportion (5–10%) of *Ixodes ricinus* ticks in eastern Poland are infected with *Borrelia burgdorferi sensu lato* which indicates a need for further studies, including the determination of *B. burgdorferi* genospecies.

Poland belongs to countries with endemic occurrence of tick-borne encephalitis with an annual number of clinical cases in the years 1996–2001 from 101–257 [4]. The foci of TBEV were identified on the territory of the Białystok, Gdańsk and Szczecin regions (northern Poland), Łódź region (central Poland), Opole region (western Poland) and Lublin region (eastern Poland) [25], but the presence of the disease focus was not confirmed in all regions by isolation of virus. For the first time in Poland, TBE virus was isolated by Przesmycki *et al.* in Opole region in 1953 [21]. 9 strains were isolated from human blood and cerebrospinal fluid and one strain from ticks. TBE virus was also isolated from *I. ricinus* in Gdańsk, capital city of the region adjacent to Baltic Sea [35]. In 1955–1957, numerous TBEV strains were isolated in the area of Białowieża Virgin Forest (Białystok region). 45 strains were isolated from small mammals, 15 strains from arthropod parasites and 6 from TBE patients [32]. In 1981 4 new strains of TBE virus were isolated in Białowieża Forest from brains of 134 mammals. No strains were isolated from 80 larval and 20 adult ticks [2]. The isolation of the TBEV strain in the present study is the first isolation of TBE virus in the Lublin region. The focus of TBE in the Radzyń Podlaski area is the fourth locality in Poland (after Kłodobok in Opole region, Gdańsk region, and Białowieża Forest in Białystok region) where the presence of TBEV in ticks was confirmed by isolation of the virus.

Mickiene [14] reported in 2001 the first isolation of TBEV strain in Lithuania from patient's serum. Kahl *et al.* [11] isolated only 1 strain of virus from 327 pools of *Ixodes ricinus* ticks in 1988 in West Berlin. The minimum infection rate obtained in our study (1.8%) is close to values found by Süss *et al.* [31] by PCR method in 1999 in eastern and southern Germany where estimated TBEV prevalence ranges were from 0.6–3.4%, depending on season and locality. Han *et al.* [8] found in 2001 by PCR method only 0.34% ticks with TBEV in endemic region of Finland.

Kicińska and Wróblewska-Mularczykowa [13] examined 530 forestry employees and 464 agricultural workers from Lublin, Olsztyn, Bydgoszcz and Koszalin regions. The highest percentage of seropositive results with TBE antigen (18.9%) was noted in forestry workers from Lublin region. The authors suggest the possibility of natural foci of tick-borne encephalitis in this region. This presumption corresponds with earlier seroepidemiological and clinical studies of our group which demonstrated the presence of endemic focus of tick-borne encephalitis in the area of Radzyń Podlaski [5, 6]. This finding has now been confirmed by the isolation of TBEV strain described in the present study. The properties of this strain will be the subject of further studies.

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